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EXPLORATION OF THE USE OF NUCLEAR MAGNETIC RESONANCE FOR THE STUDY OF RICIN TOXICITY IN CELLS

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14. ABSTRACT This report is a required report for the U.S. Army Edgewood Chemical Biological Center In-House Laboratory Independent Research project "Cell Toxicity by NMR." Traditional assays lead to IC_{50}/EC_{50} values based solely on cell survivability. However, additional information is desirable for understanding a toxin's total effects. Complex cellular metabolite mixtures can be analyzed by nuclear magnetic resonance (NMR) with the aid of statistical methods such as chemometrics analysis (CA). However, the use of NMR-CA for cell toxicity to date has required model-building with IC_{50}/EC_{50} values obtained from conventional cytotoxicity assays. Here we report on an exploratory study to determine the feasibility of using NMR-CA independently for the study of ricin toxicity in BALB/c 3T3 Murine Fibroblasts (CCL-163 American Type Culture Collection [ATCC], Manassas, VA) by analysis of the cell growth media alone. This work involves principal component analysis (PCA) as a chemometrics analysis tool. We have found that NMR-PCA readily distinguishes between multiple-component media samples from the same preparation that had cells grown in them from those that did not have cells grown in them. We also have preliminary evidence that this procedure will be able to identify differences between media from cells dosed with varying concentrations of ricin.

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PREFACE

The work described in this report was authorized under Project No. 61110191a00, In-House Laboratory Independent Research. This work was started in March 2008 and completed in October 2008.

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CONTENTS

FIGURES

TABLE

EXPLORATION OF THE USE OF NUCLEAR MAGNETIC RESONANCE FOR THE STUDY OF RICIN TOXICITY IN CELLS

1. INTRODUCTION

Traditional cell toxicity assays lead to an IC_{50} or EC_{50} value based on the determination of cell death. A method that provides information in addition to survivability regarding the effect of a toxin on the cell is desirable for understanding the total effects of the toxin. Complex cellular metabolite mixtures can be analyzed by nuclear magnetic resonance (NMR) with the aid of statistical methods such as chemometrics analysis (CA). When combined with CA, NMR may provide information in the form of biomarkers, affording detailed biochemical information not available through typical cell assays, while also allowing for the determination of EC_{50} or IC_{50}. An NMR-based CA procedure is also expected to have advantages including: no dyes or labeled compounds; the analysis of dosed cells with little sample preparation; and no additional washes or incubations after the dosing period. Here we report on an exploratory study to determine the feasibility of using NMR-CA independent of conventional cytoxicity assays for the study of ricin toxicity in BALB/c 3T3 Murine Fibroblasts (CCL-163 American Type Culture Collection [ATCC], Manassas, VA) by analysis of the cell growth media alone. This work involves principal component analysis (PCA) as the chemometrics analysis tool.

2. METHODS

Note: Ricin is extremely toxic. Its use is controlled under the Biological Select Agents and Toxins program in the United States by the Centers for Disease Control and Prevention (Atlanta, GA). Handling of ricin should follow strict safety procedures determined in collaboration with the safety office of the research laboratory's organization.

2.1 BALB/c 3T3 Murine Fibroblasts.

The fibroblasts (CCL-163 American Type Culture Collection [ATCC], Manassas, VA) were passaged a minimum of three times after thawing prior to testing. Ninety-six well plates were seeded at 2.5×10^3 cells per well and maintained in culture at 37 °C in a humidified atmosphere of 5% CO_2 in air for 24 hr prior to treatment.

2.2 Ricin Preparation.

The *Ricin communis agglutinin* II (ricin) stock solution was prepared by dialyzing ricin (Vector Laboratories, Burlingame, CA) into 10 mM sodium phosphate buffer (pH 7.0, PB) over a period of ~ 24 hr with gentle stirring using three volumes of ~ 600 mL PB. Dialysis was carried out on ice using regenerated cellulose or cellulose ester Dispodialyzers® (Spectrum Laboratories, Rancho Dominguez, CA) with 5000 or 8000 molecular weight cutoff. Ricin stock solutions were stored at 0–4 °C. Stock solution concentration was determined at 25 °C by absorbance of a stock solution dilution using a JASCO Model J-810 Spectropolarimeter (JASCO Analytical Instruments, Easton, MD) equipped with a Peltier thermoelectric temperature control system (PTC-423S). Fifteen absorbance measurements on the solvent solution without any protein were recorded at intervals of 1 s and averaged. Fifteen measurements were then

recorded on the sample and averaged. The blank average was subtracted from the sample average. The concentration was calculated using Beer's Law with $E^{0.1\%}_{280nm} = 1.4$[1]. The ricin was sterile filtered prior to use in the cell cultures.

2.3 Ricin Exposures.

Exposures were performed 24 ± 2 hr after seeding the 96 well plates. Eight different concentrations of ricin were prepared by diluting the ricin in cell culture medium. The diluted ricin was then added to 6 wells (per concentration) in each of 8 plates (Ricin Cells). These plates also included Vehicle Control wells that contained cells to which a 1:1 ratio of growth medium and chemical dilution medium was added (i.e., the wells contained cells, but no ricin). After ricin exposure, the plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air for 48 hr. Four additional 96 well plates (Ricin Blanks) that had not been seeded with cells also received ricin concentrations identical to those used to expose the plates containing cells. These plates also contained Media Blanks (no ricin, no cells). These plates were incubated in the same manner as those with cells.

2.4 NMR Sample Preparation.

Collection of NMR samples was done 48 hr ± 0.5 hr post-exposure. A total of 76 samples were collected. For each Ricin Cells sample, medium from each of the 6 wells of a given ricin concentration from one plate (8 total concentrations per plate) were pooled along with two additional wells (total of 8 wells) from duplicate plates to provide adequate volume for one NMR sample. A total of 6 samples for each ricin concentration were collected (Ricin Cells) along with six samples of Vehicle Control exposed medium. Corresponding samples (two samples per concentration, 8 concentrations) were also collected using the four 96 well plates that were exposed to ricin, but that contained no cells (Ricin Blanks), along with six Media Blank samples. After collection, all samples were filtered using a 0.2 µm sterile syringe filter to remove any possible remaining cell debris from the pooled medium. Samples were quick-frozen on liquid nitrogen and stored at -80 °C. Frozen samples were later thawed to room temperature, concentrated to near-dryness using a Centrivap (Labconco, Kansas City, MO), and stored at -20 °C until NMR analysis. The samples were separated into 4 batches (Batches 1-4) of 19 samples each for drying because the centrivap rotor could not accommodate them all. Each Batch contained samples representing the range of ricin concentrations from the Ricin Cells samples, samples representing at least three of the ricin concentrations from the Ricin Blank samples, at least one Vehicle Control sample, and at least one Media Blank sample.

2.5 NMR Experiments.

On the day of analysis, each sample was dissolved in 602 µL D_2O containing 0.5 mM 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS) and 0.01 % NaN_3 (chemicals from Sigma-Aldrich, St. Louis, MO). Samples were randomly taken from differing Batches on different days of analysis. One-dimensional proton NMR experiments were carried out on a Varian UNITY Inova 600 MHz NMR spectrometer at a regulated temperature of 25 °C. Experiments used 16K complex points, 1.00 s relaxation delay, 2.37 s acquisition time, and 6913.8 Hz spectral width. Water suppression was carried out with a soft pulse at the water resonance for 2.00 s.

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[1] Shimoda, T.; Funatsu, G. Binding of Lactose and Galactose to Native and Iodinated Ricin D. *Agric. Biol. Chem.* **1985**, *49*, 2125-2130.

2.6 Chemometrics Analysis.

The NMR spectra were initially processed with *NUTS*[2] software. The NMR free induction decays (FIDs) were converted to the frequency domain using fast Fourier transformation. The transformed spectra were individually phased, baseline corrected, and referenced to DSS at 0.00 ppm. The data was reduced by segmenting the spectra from 0.2 to 10 ppm into 0.04 ppm regions, which were integrated. The water resonance region of 4.5 -5.0 ppm was excluded. The resulting 233 values were normalized to allow for comparison among the different spectra. The data sets were exported to *The Unscrambler*[3] software for principal component analysis (PCA) and cluster analysis. Prior to analysis, the data sets were scaled using mean center scaling.

3. RESULTS AND DISCUSSION

3.1 NMR Experiments.

Proton NMR experiments were carried out on 76 samples as summarized in the Table below. A typical 1-dimensional NMR spectrum obtained for cell media on which samples were grown, is shown in Figure 1.

Table. Summary of Samples analyzed by NMR

Sample Type	Ricin Concentration (µg/mL)	# of Duplicate Samples
Ricin Cells: media, cells grown, ricin		
Concentration 1	6.0000×10^{-03}	6
Concentration 2	4.0816×10^{-03}	6
Concentration 3	2.7766×10^{-03}	6
Concentration 4	1.8889×10^{-03}	6
Concentration 5	1.2849×10^{-03}	6
Concentration 6	8.7410×10^{-04}	6
Concentration 7	5.9463×10^{-04}	6
Concentration 8	4.0451×10^{-04}	6
Vehicle Controls: media, cells grown, no ricin	---0---	6
Ricin Blanks: media, no cells grown, ricin		
Concentration 1	6.0000×10^{-03}	2
Concentration 2	4.0816×10^{-03}	2
Concentration 3	2.7766×10^{-03}	2
Concentration 4	1.8889×10^{-03}	2
Concentration 5	1.2849×10^{-03}	2
Concentration 6	8.7410×10^{-04}	2
Concentration 7	5.9463×10^{-04}	2
Concentration 8	4.0451×10^{-04}	2
Media Blanks: media, no cells grown, no ricin	---0---	6

[2] *NUTS*, version 2D; Acorn NMR, Inc. Livermore, CA. 2004.
[3] *The Unscrambler*, version 9.6; CAMO Software AS. Oslo, Norway, 2006.

3.2 PCA Analysis.

In brief, PCA calculates a set of descriptors, the principal components (PCs) that are linear combinations of the original variables, the parts per million binned regions in this case, with each PC orthogonal to the others. The first PC, PC1, explains the most variance in the data set whereas each successive PC explains the most variance remaining after the previous PC. A plot of PC1 versus PC2 is a two dimensional representation of the complete data set. A graphical separation between groups of data points on a PC scores plots indicates a toxicity-induced variation between the groups of spectra. Differences may be a result of multiple changes in the NMR spectra. The ppm regions responsible for the variation of the data points can be identified using a second graphical tool, the loadings plot.

An initial PCA analysis was carried out using all 76 samples. The analysis was carried out on binned spectra with a bin width of 0.04 ppm and covered the 0.20 – 10 ppm spectral region. The water peak region (4.5 to 5.0 ppm) was excluded from the calculations. Figure 2 shows the PC2 versus PC1 scores plot. No outliers were removed for this first set of calculations. Other than the obvious outliers (red "RC" points, left side of plot), most noticeable is a clear separation based on the Centrivap Batch number. This is accounted for by the fact that the Centrivap developed a vacuum leak that was not initially noticeable, but that increased over time, with the result that the dry-down time successively increased for each Batch dried until the leak was noticed. The separation in the scores plot follows a trend corresponding to the length of dry-down time (Batch 4 = shortest, Batch 3 = longest).

Figure 3 shows the PC4 versus PC3 scores plot from the same analysis (all 76 samples, no outliers removed). Here, separation occurs between the media in which cells were grown and the media in which no cells were grown but which were otherwise incubated and processed in the same manner. Because of the obvious effect of the dry-down time on the analysis noted in the PC2 versus PC1 discussion above, PCA was next carried out on individual batches to see if the separation noted here in the PC4 versus PC3 scores plot would improve. Figure 4 shows the resulting PC2 versus PC1 scores plot for the PCA of Batch 1, this time with outliers removed. The separation is now at the PC2/PC1 level. The lactate chemical shift is the primary contributor to the separation. The concentration of lactate (1.33 ppm NMR doublet, see Figure 1) is higher relative to other metabolites in the media that had cells grown in it compared to the Blanks. The fact that the Blanks with and without ricin do not separate from each other indicates that the separations relate to cell activity rather than to the low levels of ricin that may be present in a sample. To complete the exploratory study, PCA was again carried out on individual Batches, this time with just the Ricin Cells and Vehicle Control samples. Figure 5 shows the resulting PC2 versus PC1 plot for Batch 4. For concentrations having more than one duplicate in the batch, the samples are grouped relative to the ricin-dosing concentration. This result is promising and indicates that there may be metabolic effects that depend on the dosing concentration.

4. CONCLUSIONS

We have found that nuclear magnetic resonance-principal component analysis (NMR-PCA) readily distinguishes between multiple-component media samples from the same preparation which had cells grown in them from those which did not have cells grown in them. In addition, we have shown that NMR-PCA detects variation in the time taken to dry down samples for NMR analysis. Finally, we have preliminary evidence that this procedure will be able to identify differences between media from cells dosed with varying concentrations of ricin.

Additional studies should include a replicate of the study carried out here, but with all samples dried down simultaneously and as quickly as possible. This change in procedure will allow for more statistically relevant PCA from which variation of specific metabolites would be expected to lead to an EC_{50} determination.

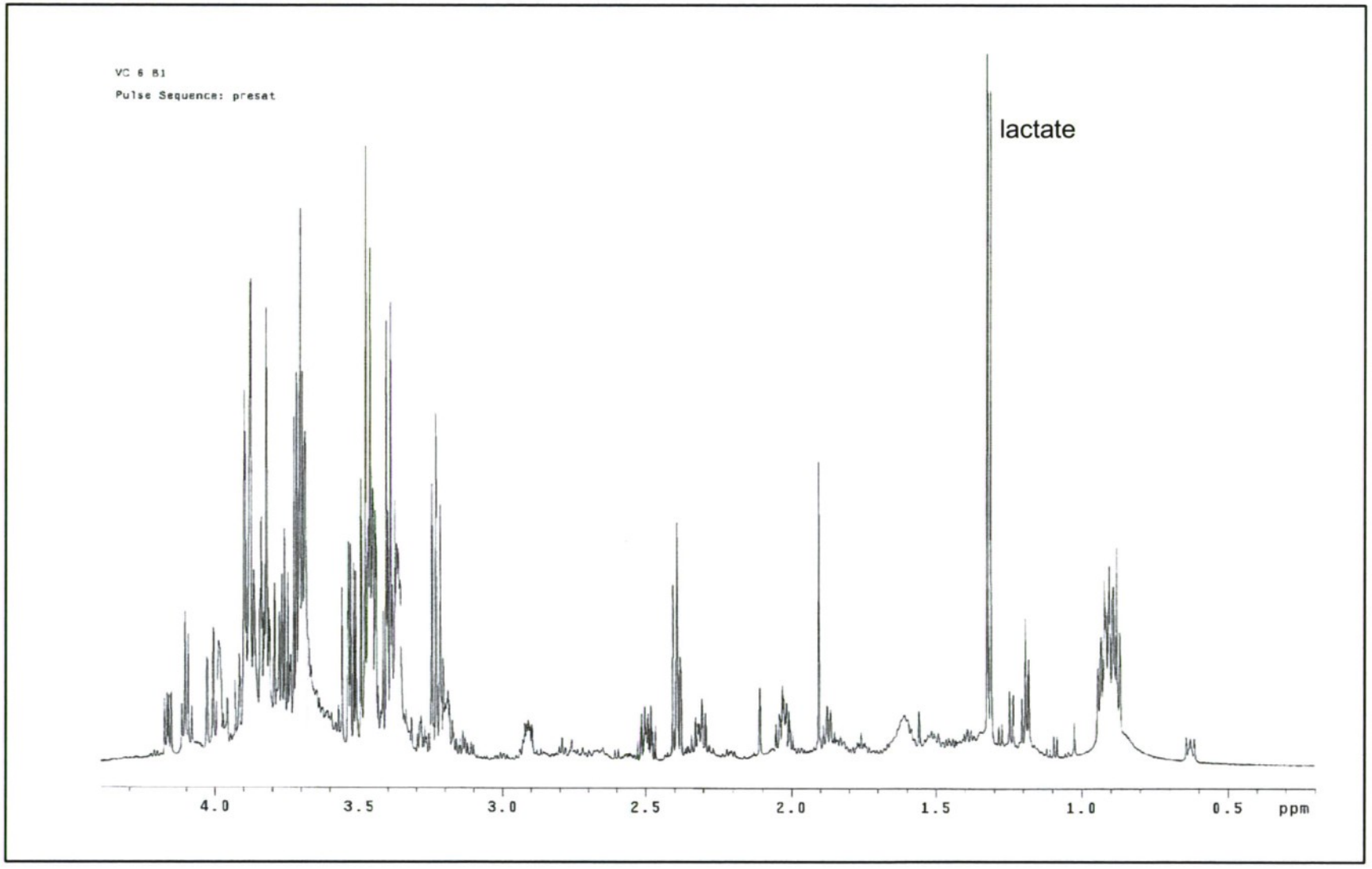

Figure 1. Proton NMR Spectrum Expanded Region (4.8-0.2 ppm) for a Vehicle Control Sample that Contains Media on which Cells Were Grown. This region contains the peaks of importance for the chemometrics analysis described.

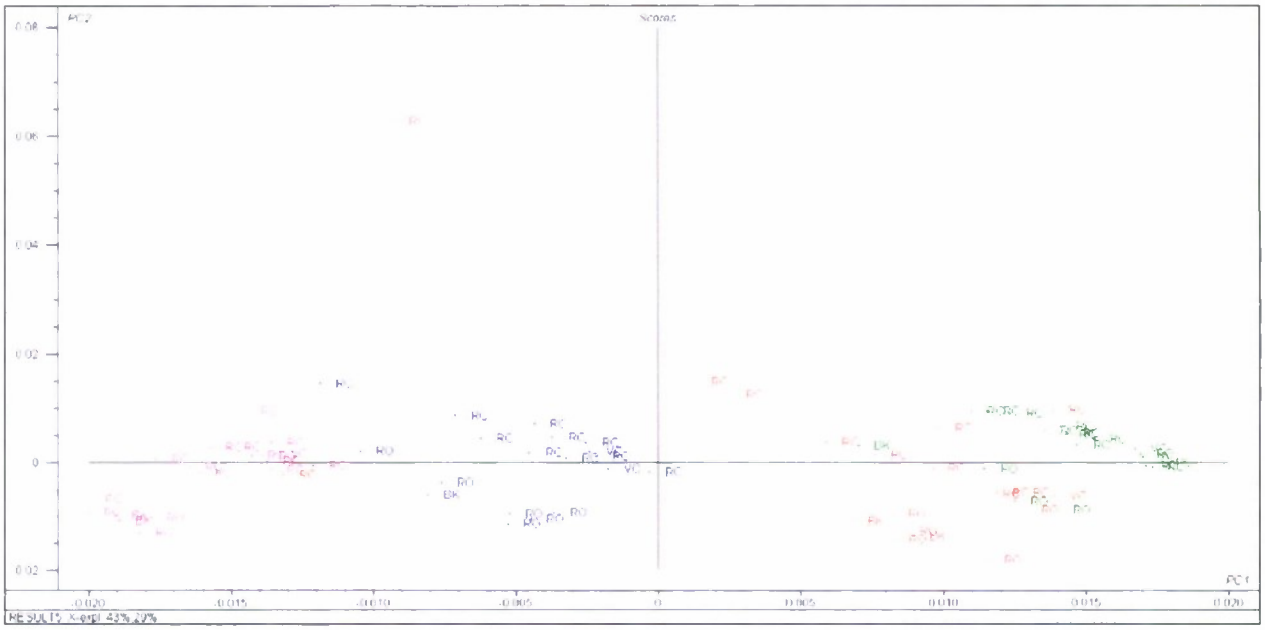

Figure 2. PC2 versus PC1 Scores Plot for PCA Involving 76 Spectra. Label Key: RC = Ricin Cells samples, RO = Ricin Blanks, BK = Media Blanks, VC = Vehicle Controls. Color Key: Batch 1 = blue, Batch 2 = red, Batch 3 = green, Batch 4 = magenta.

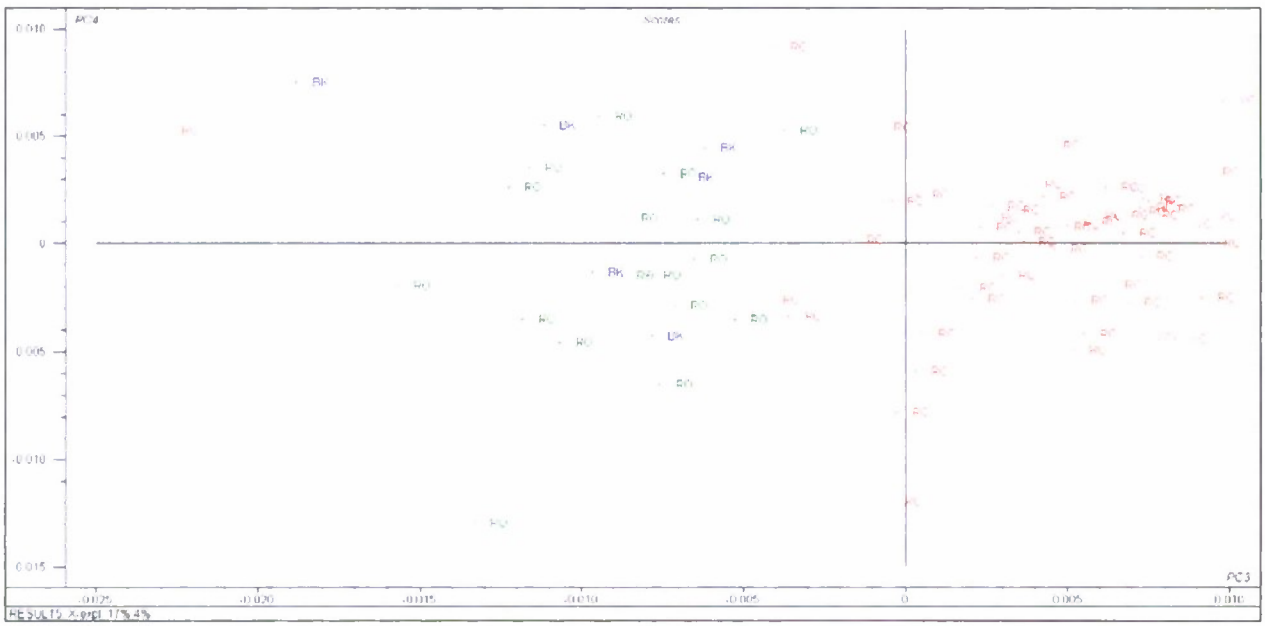

Figure 3. PC3 versus PC4 Scores Plot for PCA Involving 76 Spectra. Key: RC = Ricin Cells samples, RO = Ricin Blanks, BK = Media Blanks, VC = Vehicle Controls.

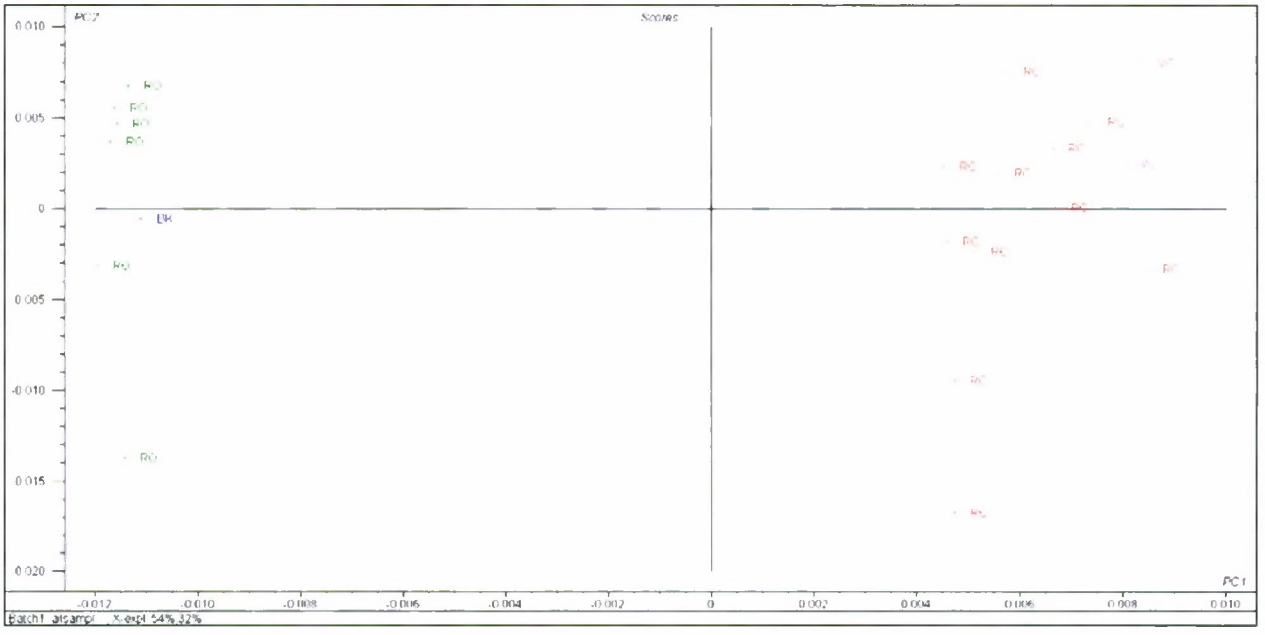

Figure 4. PC2 versus PC1 Scores Plot for PCA Involving Batch 1 Spectra. Key: RC = Ricin Cells samples, RO = Ricin Blanks, BK = Media Blanks, VC = Vehicle Controls.

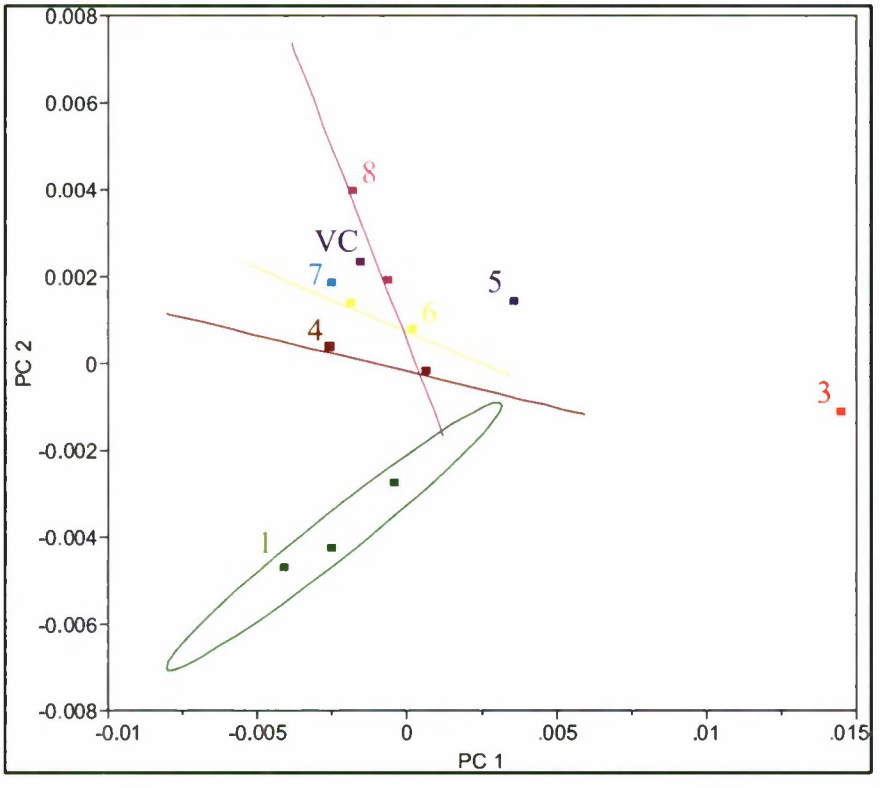

Figure 5. PC2 versus PC1 Scores Plot for PCA Involving Batch 4 Spectra. The numbers correspond to the ricin concentrations used for the Ricin Cells samples, where #1 is the lowest concentration and VC is the vehicle control sample. The ellipses are at the 0.95 confidence level.